

**THE MASS PRODUCTION OF CONIFER TREE HYBRIDS
SUSPENSION CULTURE SCALE-UP FOR DOUGLAS-FIR
AND LOBLOLLY PINE**

Project 3223

**Report Six
A Progress Report
to
MEMBERS OF THE INSTITUTE OF PAPER CHEMISTRY**

July 17, 1978

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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THE MASS PRODUCTION OF CONIFER TREE HYBRIDS

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AND LOBLOLLY PINE

SUMMARY

Our sixth report to the industry for the term 1977/1978 deserves special attention. The goal of the mass production of conifer hybrids and our progress in this area is seen as a transfer of information, technology, and manpower to the industry. This is in keeping with the Institute's aim of "conducting research in fundamental and applied areas of long-term interest to the paper industry and to development of scientific generalists with pragmatic emphasis on the usefulness of graduate education."

Our project is unique in the industry. It deals with innovation and the application of the new biology in ways that insure, in the face of expected increased demands, the supply of wood for the paper industry. Opportunities are now evident to capitalize on existing genetic gains on a massive scale and to bioengineer trees with traits specifically suited to the end uses of trees of concern to the members. Clearly our research must become more client-oriented. For this challenge we have established a revised research plan and emphasized the transdisciplinary nature of the project. By contrast with the operations of most other agencies, we offer the industry input to our project and representation through the Program Advisory Committee for Forest Genetics and a solid long-term commitment to the goals established through interactions between Members and the Institute.

In the past, the Institute has demonstrated competence in the area of tree biology. In 1969, the first test tube tree, a triploid aspen, was grown

from callus. Recent additions to the staff include scientists who have produced the first tree from cell suspensions and who have had experience in regenerating plants of agricultural value. Graduates of the Institute, knowledgeable in tissue culture, have moved to the paper industry and have established their own projects.

Over the past year, we feel that the achievements are impressive. They provide a landmark in the scale up and control over the development of Douglas-fir and loblolly pine cells in suspension culture. Currently the Institute has 20 liters of Douglas-fir cells in suspension. One hundred liters would be sufficient to reforest 100,000 acres at a 10 x 10 ft spacing should 90% of the cells and clumps develop into plantlets. The cells can be maintained indefinitely on a simple, chemically defined nutrient medium. As for loblolly pine, we have about 10 liters of cells. This represents a major breakthrough because, in the past, loblolly pine has been difficult to maintain indefinitely in suspension.

The highlight of this year's report is seen in the demonstrated controlled development of cells of both species in suspension culture. For the first time the growth of the daughter cells has led not to the production of more nondescript cells but to the formation of small clumps that show promise of developing into trees by two routes. One route shows signs of repeating the normal process of embryo development. The second leads to the specific production of organs bypassing the steps in embryogenesis. To our knowledge, most other agencies that have established cell suspensions of conifers have not achieved this degree of control and their cells merely produce more nondescript cell populations. We have evidence that not less than 80% of our cells in suspension grow to produce compact spherical structures that can differentiate and produce the structural elements of primary xylem. Results have been repeated for Douglas-fir and loblolly pine.

The challenge is clear and has never been more exciting. We must understand that so far our progress reflects the predetermination of parameters needed to grow and develop the suspension cultures. There is no doubt in our view that a better degree of control over the reactions in the flasks is required. For this work, we look to support from the member companies. Our team has already started on the design of a new culture system that we expect will be a prototype for the industry. Visits with some of our clients indicate that our technology can be eventually integrated directly with operational programs that generate containerized plants of genetic gain and of specific traits that would better serve the paper and forest products industry.

RESEARCH OPERATIONS

INTRODUCTION

For decades, research with forest trees has lagged behind advancements in agriculture and medicine. Forestry research looked to these fields for directions and developments that could help domesticate and improve trees, especially the coniferous species. This fact was recognized as early as 1929 when a special task force was set up to evaluate research in forestry. The observation was made that research in forestry would continue to lag behind agriculture until a special physiological technique was devised to allow the study of trees in the laboratory.

Today, we have this technique. Through cell and tissue culture, new technologies have developed that will revolutionize forestry. They have already revolutionized the field of horticulture. Currently, nearly 70 establishments are applying tissue culture in their commercial greenhouse operations. In forestry, it is a most dramatic demonstration to the novice that a few live cells taken from a species as large as Douglas-fir could be grown and multiplied by the millions like microorganisms in a few liters of nutrient medium. Furthermore, the daughter cells that grow can be controlled so that the tissues generated grow in an organized fashion. This is the subject of this year's progress report. We consider the progress a breakthrough and a most encouraging development for member companies. We now have a potential forest in a flask!

Events leading to our progress must be seen in the recent rise in new information in this field (Fig. 1) with the recognition that the Institute has a long-term commitment in this field and will continue to exhibit leadership in three main areas: First, in the rise of knowledge; second, in the contribution

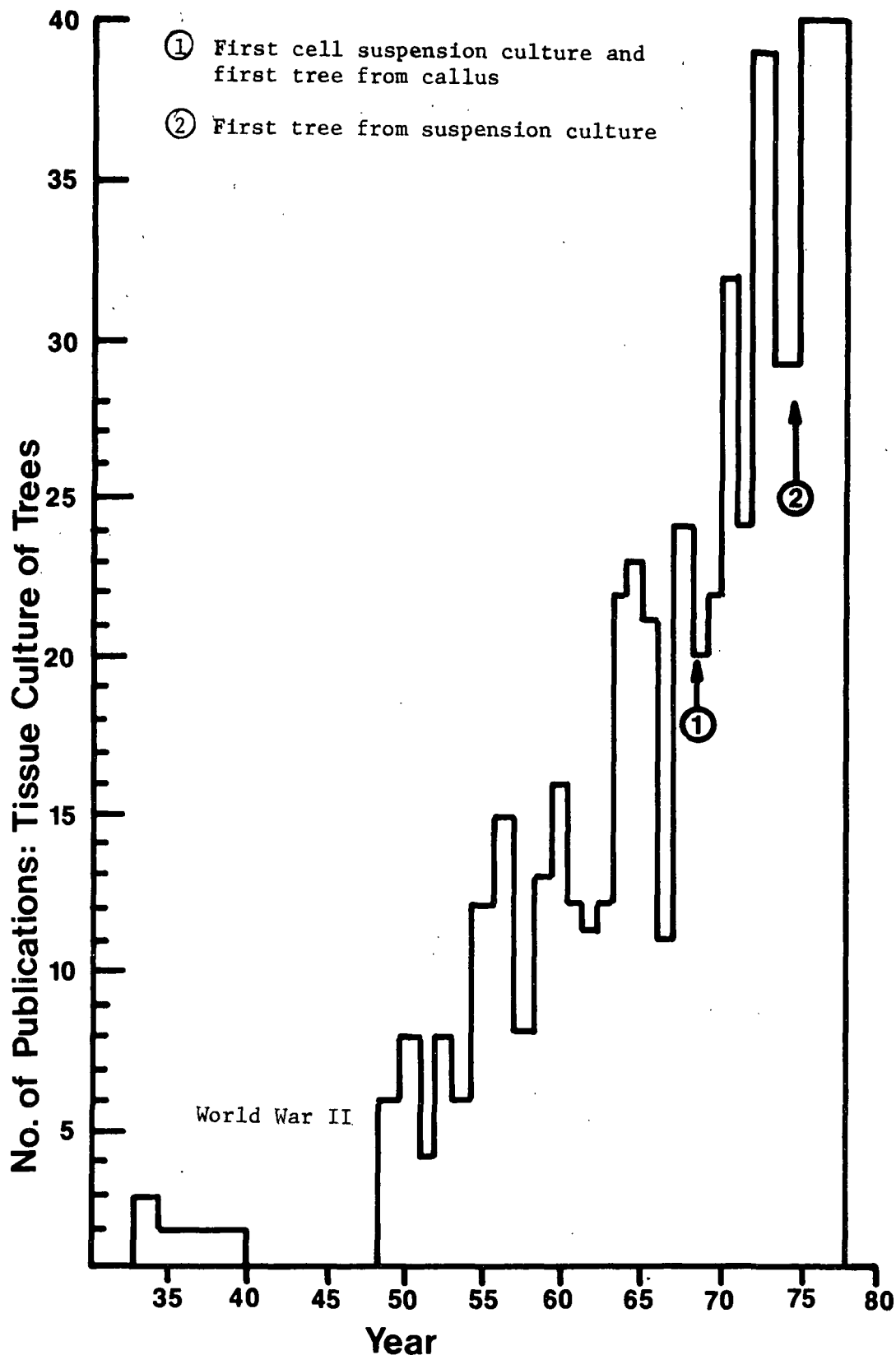


Figure 1. Number of Publications on Cell and Tissue Culture of Forest Trees as a Function of Time. Arrow 1 Points to the Production of the First Tree (Aspen) from Callus Tissues and the Establishment of First Cell Suspension Cultures of Conifers. Arrow 2 Points to the First Tree (Elm) from Suspension Cultures and to the Production of Pine from Callus

to new technologies; and, third, to the manpower base that could be used to exploit the newly created opportunities for tree improvement.

This year's progress is seen against the background of a transition in project leadership and a new long-term research plan which is partially outlined in this report. The overall research effort has been transdisciplinary. New opportunities for interactions with our clients have been identified. Reports from team scientists, expert in certain disciplines, relate their work to specific subobjectives of the plan. Several of the objectives have already been met.

We ask that individuals and decision makers who are interested in our project take advantage of the requested information exchange at the end of our report. Please let us know of your interest so that we can better serve you.

RESEARCH PLAN

General

From scenarios among member industries it is clear that the expanding demands for forest products are expected to outstrip the supply of wood. Currently, there are 1.25 billion trees planted in the U.S.A. and it is the most practical approach that these trees reflect the industry's attempt at domesticating trees. This includes maximizing the genetic gains and developing methods to evaluate the progeny. Unfortunately, not all of the existing technologies allow for the mass propagation of the specific gains and none improve upon ways that new traits can be introduced to better meet the needs of industry. It is our view that these two fundamental constraints can be overcome by cell and tissue culture technologies. Our report will show that tree cells grow rapidly like microorganisms and produce the massive numbers of prospective plantlets needed by industry. The proviso is that these cells are totipotent, i.e., have

the information needed to grow back into trees. We have evidence that the concept of totipotency will be proven accurate. Indeed, in the cloning procedures used by industry to date, there has been no evidence to believe the contrary nor has there been any indication that the basic genetics of the propagules have been altered. Another advantage of our new technology is that the cellulose and pectin walls of the cells can be removed by enzymes to produce naked cells or protoplasts (Fig. 2). At the Institute's laboratories, conifer protoplasts from different genetic sources have been shown to fuse. These observations open the door for new opportunities in tree genetics that may someday permit the engineering of trees suited to industry's needs.

The Institute's objectives include production of technical manpower for the 1980's. The long-term commitment to excellence is in keeping with the development of new technologies in answering industry's needs, particularly in the resource base. The project's technologies are basically of two types. The first is biomimetic, i.e., innovations for industry based on principles that mimic or imitate natural events. The second involves bioengineering, i.e., the introduction of new innovations into biological systems that allow the industry to get from its resource base, more from less, quality from quantity and provides systems that reduce the risks of growing trees ill-suited for the production of paper or other forest products.

The project's objectives are first, to provide our clients in industry with the information, technology, and manpower specifically related to the mass propagation of coniferous trees to maximize genetic gains. The second objective is to improve the genetic base by the creation of new hybrids suited to industry's needs through bioengineering using protoplasts and the newer developments in molecular biology and genetic engineering.

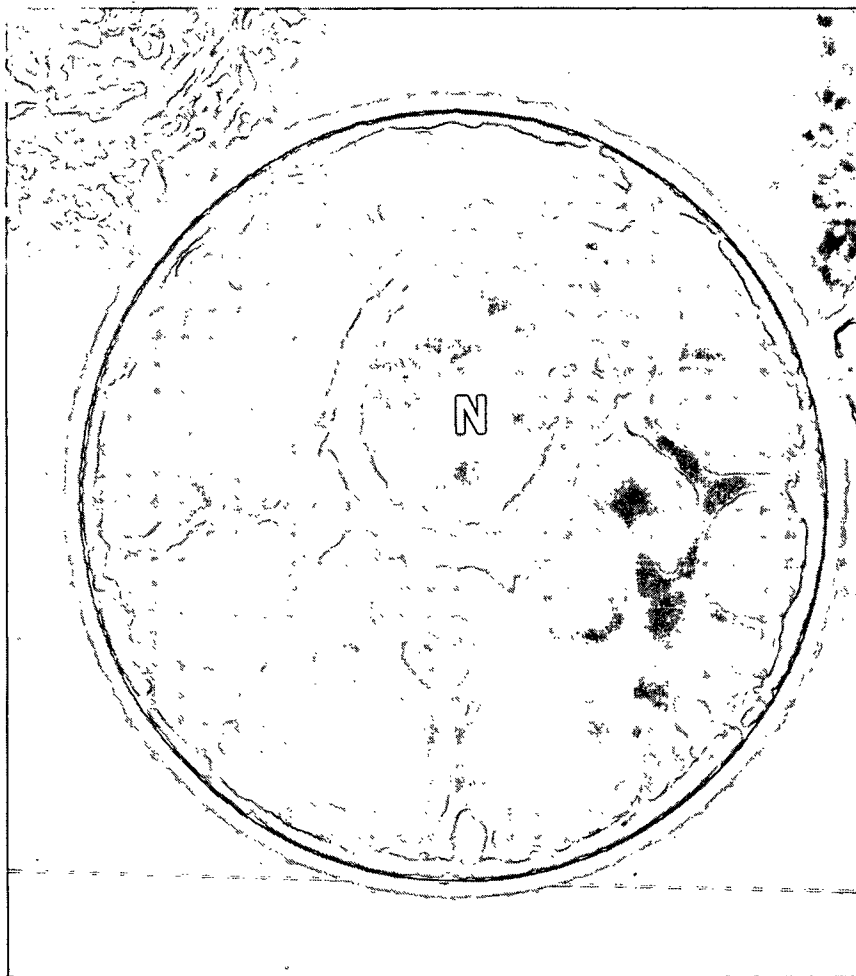


Figure 2. Protoplast of a Douglas-fir Cell. The Cell Wall has been Enzymatically Removed and the Protoplast is Ready for Fusion with Other Protoplasts. N. Nucleus Containing the Genetic Information of the Tree from Which the Protoplast was Derived. X500

The team's output will be seen as a spectrum of innovation based within the Institute's mandate. The initial phases involve the transfer of scientific information and manpower to the industry after tests to show that the information is sound and valid. We are now well into this first phase and are entering a new one where the principles are being applied in an engineering sense to meet our objectives. The project has moved away from the earlier empirical studies and is engaged in the design of systems where greater control is imposed on the development of cells. We have already learned that prototype technologies that could be built at the Institute with industry's support could support existing tree farms especially those of the larger companies. As methodology is refined, there is no doubt that the smaller companies would also benefit.

In the future, we look to the field-testing of our prototype technologies and the provision of systems required for the management of these technologies. It is important to understand that, at each stage, member companies have the opportunity to contribute to the design of our product through the Research Advisory Committee in Forest Genetics and through more active consultations with our experts.

Clearly the research plan is long term, complex and transdisciplinary. The plan is designed for industry and represents a commitment to the Institute's membership. It should also be evident that a number of critical subobjectives should be identified. These will be listed below. Details are available through consultations with the Research Advisory Program Committee for Genetics drawn from member companies.

Essentially, the plan is composed of 12 subobjectives. Phase One consists of subobjectives one to six. Some of these have already been attained and the goals validated by repeated experimentations. Our 12 subobjectives are:

1. Selection of the genetic base best suited to client needs.*
2. Establishment of cell and tissue culture systems that allow for the mass propagation of coniferous trees.*
3. Control the mass propagation of totipotent cells.
4. Development of a theoretical basis for developmental and morphogenetic events.
5. Development of indicators of progress and change agents that ensure the fidelity of our systems and facilitate rapid attainment of our goals.
6. Control the morphogenesis of large populations of cloned cells.
7. Establish the production of protoplasts.
8. Control the development and morphogenesis of protoplasts.
9. Produce new hybrids through protoplast fusion or through recent developments in genetic engineering.
10. Recover individual trees and specific gains through cell and tissue culture technology.
11. Repeat Step 10 on a mass scale in cooperation with our clients.
12. Transfer our technology information and manpower to member companies where appropriate.

Plans for the next one-year term are presented and validated through consultations with the Chairman of the Research Advisory Program Committee for Forest Genetics.

*These subobjectives have been attained. Considerable progress has been made in the remainder and will be identified in future annual reports.

PERSONNEL 1977-1978

Principal Investigators

Dr. D. J. Durzan: Project Leader (Ph.D., Cornell; B.S., McMaster)

Don is the newest member of the team and comes from Environment Canada where he served as Senior Advisor, Research Director, and Research Scientist over a period of 16 years. He has published over 100 scientific articles in environmental sciences, analytical biochemistry, tree biology, insect physiology and biochemistry, nutrition, seed physiology, and tissue culture.

Dr. D. W. Einspahr*: Forest Geneticist (Ph.D., M.S., B.S., Iowa State)

Dean is Division Director of the Forest Biology Section and provides the genetic input into our project. Dean has published over 50 papers in forestry journals and books and has considerable experience with the pulp and paper industry.

Dr. M. A. Johnson: Biochemist (Ph.D., Oregon State; M.S., B.S., North Dakota State)

Morris provides the biochemical basis for our observations and studies molecular mechanisms that predict and control morphogenesis in our tissue culture systems. He has at least 12 publications.

Dr. R. A. Parham*: Fiber Scientist (Ph.D., Syracuse; M.S., B.S., North Carolina State)

Pete provides support on the interpretation of cell wall structures and service work with the transmission and scanning electron microscopes. Pete has over 30 publications to his credit.

*Part-time or service commitment.

Dr. L. L. Winton: Tissue Culture Specialist and Cytologist (Ph.D., M.S., Minnesota; B.S., Univ. of California, Berkeley)

Lawson is a long-standing member of the team with over 30 publications dealing largely with tissue culture systems. Lawson maintains the cell suspensions and studies embryogenesis and organogenesis of Douglas-fir.

Research Fellows and Associates

J. Carlson*: Biochemistry, Research Fellow (B.S., Minnesota)

G. Dawson: Chemistry, Research Assistant (B.S., Northland College, Wisconsin)

H. Kaustinen*: Fiber Science, Research Fellow

Dr. F. Hsu: Post-doctoral fellow in Biochemistry (Ph.D., Yale)

S. Verhagen: Tissue Culture Research Assistant

Dr. K. Waithaka: Post-doctoral fellow in Cell and Tissue Culture (loblolly pine) (Ph.D., Wisconsin)

Student Research

J. Bobalek: Ph.D. program; Advisor: M. Johnson

B. Cann: A-291 Independent Study; Advisor: D. J. Durzan

M. Hanegraaf: Ph.D. program; Advisor: D. J. Durzan

S. Monroe: A-291 Independent Study; Advisor: L. Winton

K. W. Robinson: Ph.D. program; Advisor: Parham/Johnson

M. Smits: Ph.D. program; Advisor: M. Johnson

RESEARCH ADVISORY PROGRAM COMMITTEE (FOREST GENETICS)

Dr. J. R. Rediske (Chairman, terminates 1978)	Weyerhaeuser, Centralia, Washington Manager, Forest Tree Improvement Research
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Dr. R. H. Smeltzer (prospective chairman)	International Paper Co., Tuxedo Park, New York Research Associate
----------------------------------------------	----------------------------------------------------------------------

*Part-time or service commitment.

Dr. R. W. Ritzert Owens-Illinois, Toledo, Ohio
Senior Biochemist

Dr. W. Carlson Crown Zellerbach, Aurora, Oregon

Two meetings with the Institute team were held in Appleton on
October 28, 1977 and on March 30, 1978.

Dr. Durzan reviewed the project with the Research Advisory Committee
(Dr. F. K. Hall, Chairman) on October 20, 1977.

RESEARCH HIGHLIGHTS

INTRODUCTION

The project remains in a transitional phase with the establishment of a new project leader, the set-up of a new laboratory, and the newly-built scale-up system for suspension cultures (Fig. 3). Working relationships among principal scientists are continually readjusted to meet the needs of transdisciplinary efforts and integrated to quickly meet our objectives. Currently we have three fully committed scientists, two service-oriented investigators, and two post-doctoral fellows.

Constraints in time and funds linked to the cost of doing research, over and above salaries, coupled with the urgency to demonstrate progress meant that several contingency studies had to be initiated. Amongst these was the first effort by the team to take cells from the newly designed system and induce growth such that the daughter cells remain bound to the initial cells in suspension rather than separate to produce more cells from nondescript callus. Our progress will show that the controlled growth was achieved with Douglas-fir and loblolly pine. Furthermore, in this effort to get PLANTS FROM CELLS, an encouraging and remarkable degree of cellular differentiation was observed. We claim that for Douglas-fir and loblolly pine our results represent a breakthrough that has transcended efforts of all laboratories inside and outside of the paper industry.

The general system formulated by our team to deal with embryogenesis and organogenesis is illustrated in Fig. 4. For each step, the principal and service-oriented investigators have developed experimental systems to deal with the scientific issues needed to meet our 12 subobjectives. The following

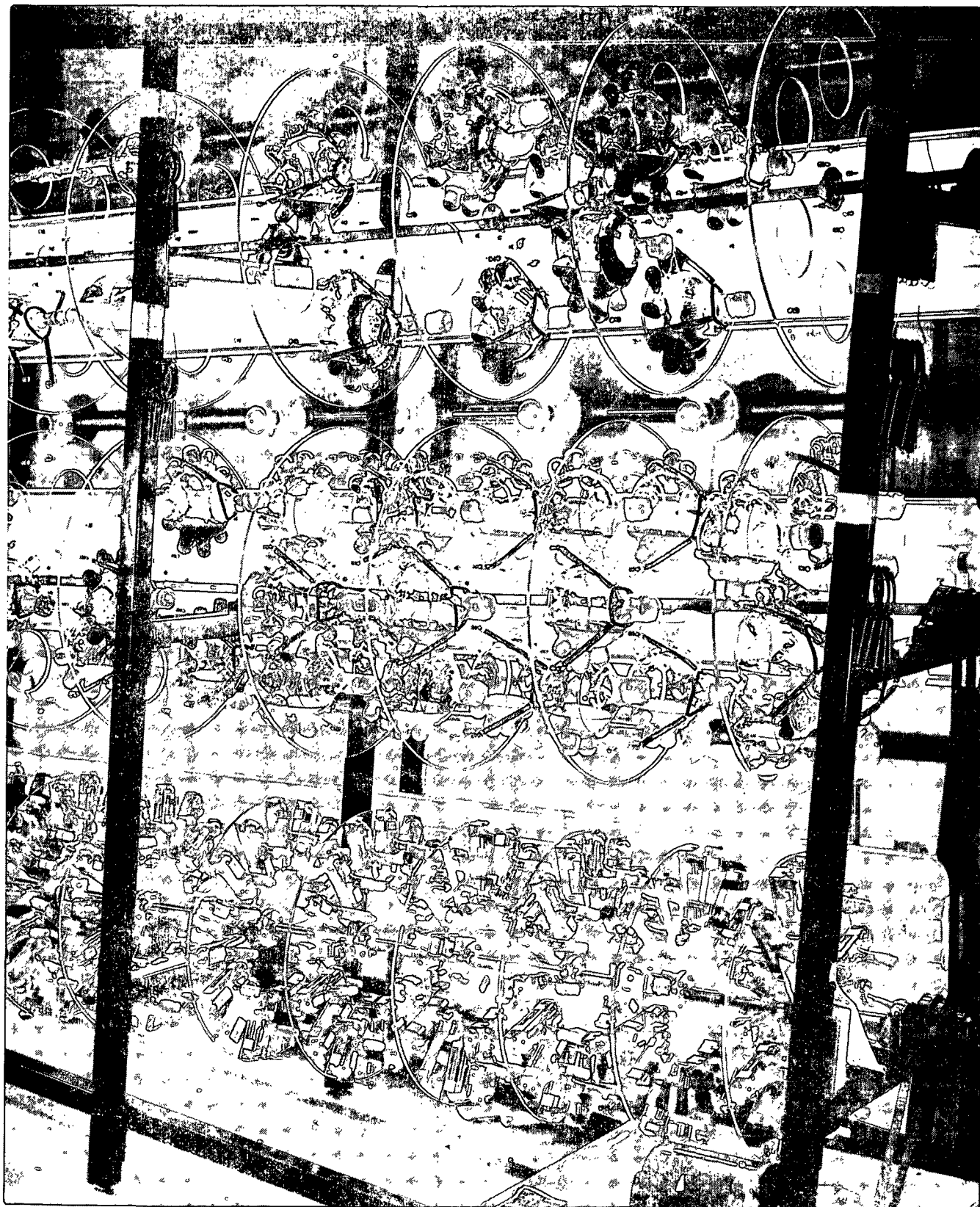


Figure 3. Apparatus for the Culture of Cell Suspensions
of Douglas-fir and Loblolly Pine

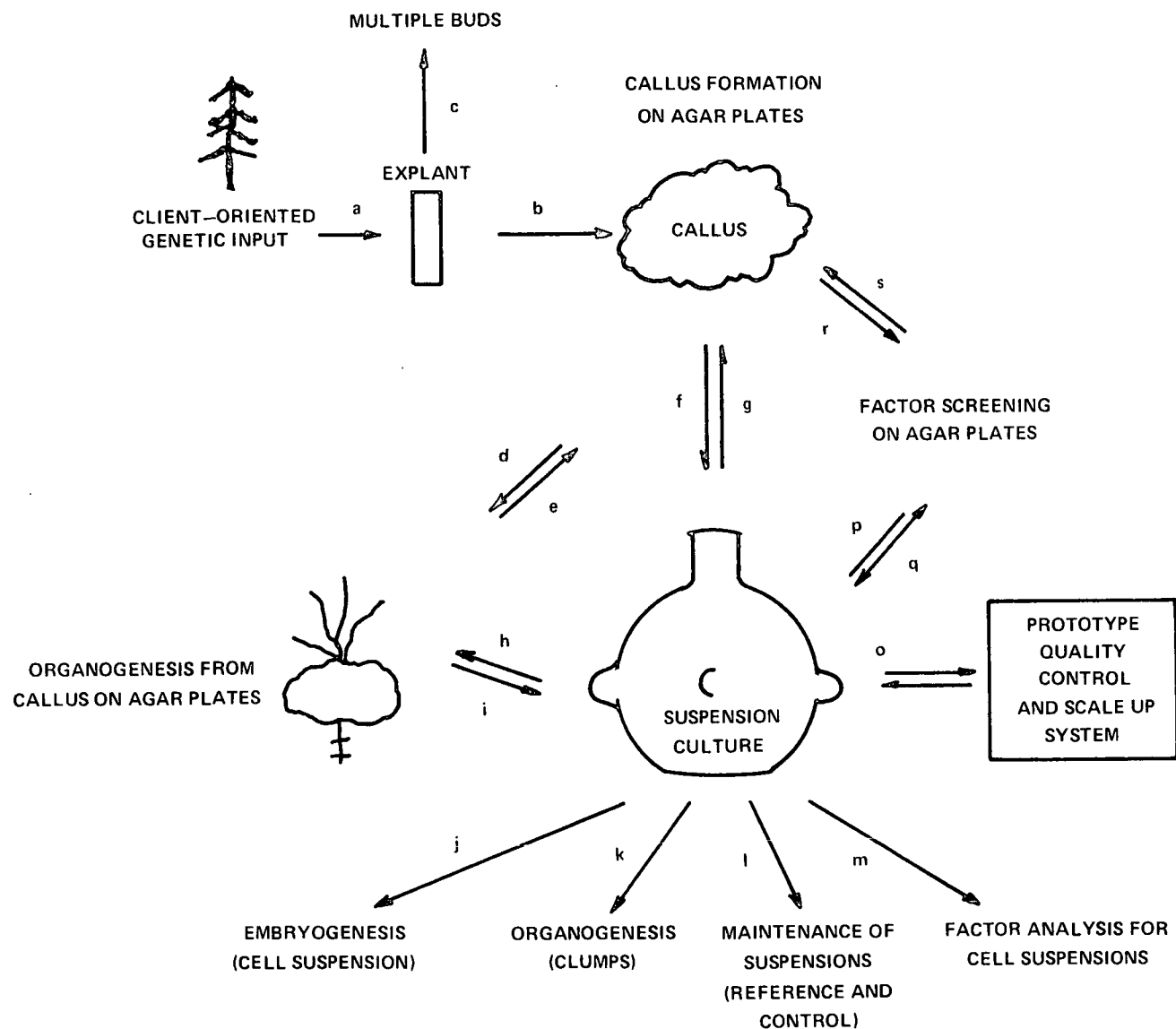


Figure 4. Orientation Model to Identify Stage at Which Experimental Data are Generated

additional developments currently under investigation will be described in more detail in future annual reports:

1. Factorial screening systems
2. Response markers for change agents
3. Innovative markers for morphogenesis
4. Isolation of stress factors induced by change agents
5. Design and synthesis of new change agents
6. Change agent detection systems
7. Identification of new promising factors controlling morphogenesis
8. New conceptual working models and prototypes
9. Totipotency evaluation systems
10. Base-line monitoring system

GENETICS

Mission

Selection of genetic base best suited to client needs.

Factors

The ultimate goal is to provide industry with a reliable procedure that allows the mass propagation of elite trees, conventionally produced hybrids and hybrids produced by protoplast fusion, thus greatly increasing the chances for a variety of types of genetic gains (growth rate, insect and disease resistance, climatic adaptability, wood quality).

Highlights

The desire to immediately attempt to capture some of the known genetic gains that exist in presently available elite trees and existing hybrids has had

to be tempered with the realization that first, a very basic and fundamentally sound cell suspension-embryoid-plantlet procedure (CSEP) needs to be worked out. Based upon the advice of the RAC Program Advisory Committee, the research team decided against working only with specific elite trees or conifer hybrids in developing the CSEP procedure and instead is using a range of genetically diverse seed sources for our biochemical investigations and the callus and cell suspension initiation work. We have also decided to temporarily pass up initiating any research on the biochemistry of fusiform rust resistance in loblolly and short-leaf pine, a potentially very fruitful research area.

The seed sources used either in the recent past or presently being used by the tissue culture program include:

Douglas-fir --

1. Seed Lot 491-15-1; Weyerhaeuser; 1500 ft elevation, east of Roseburg, Oregon, Umpqua River.
2. Seed Lot 251/256; Brown Seed Co./ 3500 ft elevation, Umpqua River drainage, Southwest Oregon.

Loblolly pine --

1. Seed Lot IPC-3223-1; International Seed Co.; Cullman County, Alabama.
2. Seed Lot IPC-3223-2; International Seed Co ; Livingston Parish, Louisiana.
3. Seed Lot IPC-3223-3; International Seed Co.; East Texas.
4. Seed Lot IPC-3223-4; International Seed Co.; South Carolina Coastal Plain.
5. Seed Lot IP-2-301-171; International Paper Co.; Seed Orchard Seed (IPC 3223-5).
6. Seed Lot UC-10-10; Union Camp Corp.; Selected tree seed.
7. Seed Lot UC-10-14; Union Camp Corp.; Selected tree seed.

Plans

Future plans include trying out the developed propagation techniques and biochemical comparisons, using several selected clones that have been produced and evaluated by cooperating organizations including Weyerhaeuser Company, Texas Forest Service and United States Forest Service. Growth rate and fusiform rust resistance are expected to be the principal parameters considered in our requests for elite trees to be used in clarifying the overall usefulness of the propagation techniques and biochemical information. Basically, the plan that has evolved regarding genetic input has been to assign the highest priority to the development of an appropriate propagation technique and to assign a very low priority to Institute production of genetically superior material.

TISSUE CULTURE

Mission

Of the 12 subobjectives in the master research plan, No. 7-11 are long-ranged in nature and involve future manipulations of protoplasts (cells without walls). Among the short-ranged subobjectives, the tissue culture laboratory has primary missions in three:

- (2) Establish cell and tissue culture systems.
- (3) Control and mass produce totipotent cells.
- (6) Control over morphogenesis.

Factors

The major factors investigated during the past year under subobjective (2) were type of vessel and environmental conditions for initiating and maintaining cultures of callus tissue on solidified agar medium or suspension cultures of cell clumps in liquid medium. Under subobjective (3), environmental and nutritional

factors were studied that permit the scale-up of cells competent to undergo morphogenesis into multigram amounts. Subobjective (6) factors included manipulation of the cell clump size and composition of freshly-added medium to allow the expression of organogenesis in large clumps and embryogenesis in small clumps.

Research Highlights

In reference to the orientation model in Fig. 4, letter (a) refers to the source of explant material. Douglas-fir (Pseudotsuga menziesii) and loblolly pine (Pinus taeda) are the only two source species at present. Douglas-fir is still preferred because it is easier to work with and comparative studies indicate many similarities between the two. However, during this past year several advances have been made in culturing loblolly pine, and in time it and other southern pines are expected to play the major role in providing explants for callus. Studies with loblolly pine will be presented later by Dr. Kimani Waithaka, Post-doctoral Research Fellow, but the remainder of this section will deal solely with Douglas-fir.

Several changes were made in regular medium 10 for more rapid initiation and growth of Douglas-fir callus from seed and seedling explants, as well as from needles, buds and other meristems from trees (Stage b, Fig. 4). Callus cultures are normally subcultured to fresh medium after four weeks.

For suspensions from callus (Stage f), the test tubes used in past years were mostly replaced with nipple flasks. Some test tubes and T-tubes are also used for screening of callus cultures or change agents for morphogenesis. One of the highlights of the year was the installation of a large rotary apparatus that will turn up to 96 nipple flasks and 192 T-tubes at 1 rpm, in a room completely controlled for light, temperature and humidity.

Another highlight was the development of a method to maintain continuous suspension cultures (Stage l). Two of three basic liquid media tested were satisfactory for maintenance, but the critical factors were the hormones, environment and physical manipulation.

By far, the most exciting results were in the control of morphogenesis in suspensions. By separating the large and small cell clumps in a suspension, each fraction could be directed to differentiate toward embryogenesis in large clumps (Stage k) or toward embryogenesis in small clumps (Stage j). Using the same technique for three basic media, repeatable and duplicated experiments gave cell differentiation that approached the formation of primary xylem elements in vascular strands. Figures 5-8 show a range of histogenesis observed in several experiments. The team has just started a definitive transdisciplinary experiment to document this control of morphogenesis, which will be reported next year.

Although most of the time was spent on organogenesis (Stage k), some preliminary work was done on the next area of emphasis, embryogenesis from small clumps (Stage j). In past years, the main problem with survival of small embryo-like structures was probably the misuse of substrate and the buildup of phenolics in the cell vacuoles, preventing elongation and resulting in death. With the interfacing of three laboratories, expertise will be combined in the form of additives recommended by the biochemistry laboratory to either prevent the formation of phenolics or to inactivate them if formed, manipulative techniques to increase survival of small cell clumps in suspensions of low densities in the tissue culture laboratory, and confirmation by the electron microscope laboratory that visual greening of cells has an ultrastructural basis.

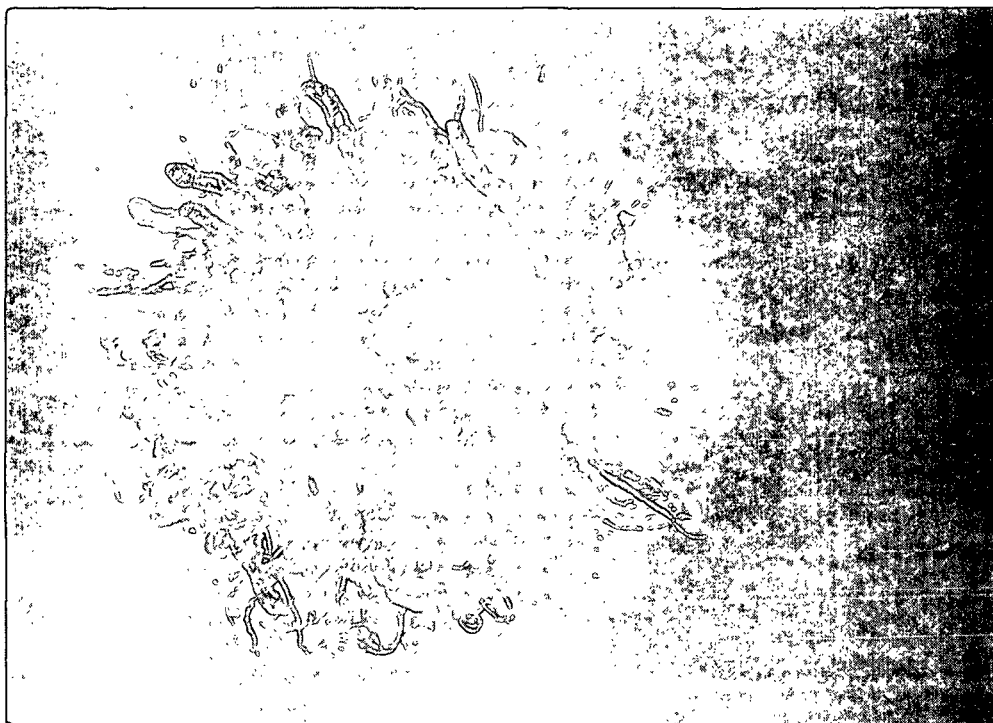


Figure 5. Light Micrographs of Organogenesis in Large Clumps of Douglas-fir Cells in Suspension Cultures. Large Clump in Phase Contrast

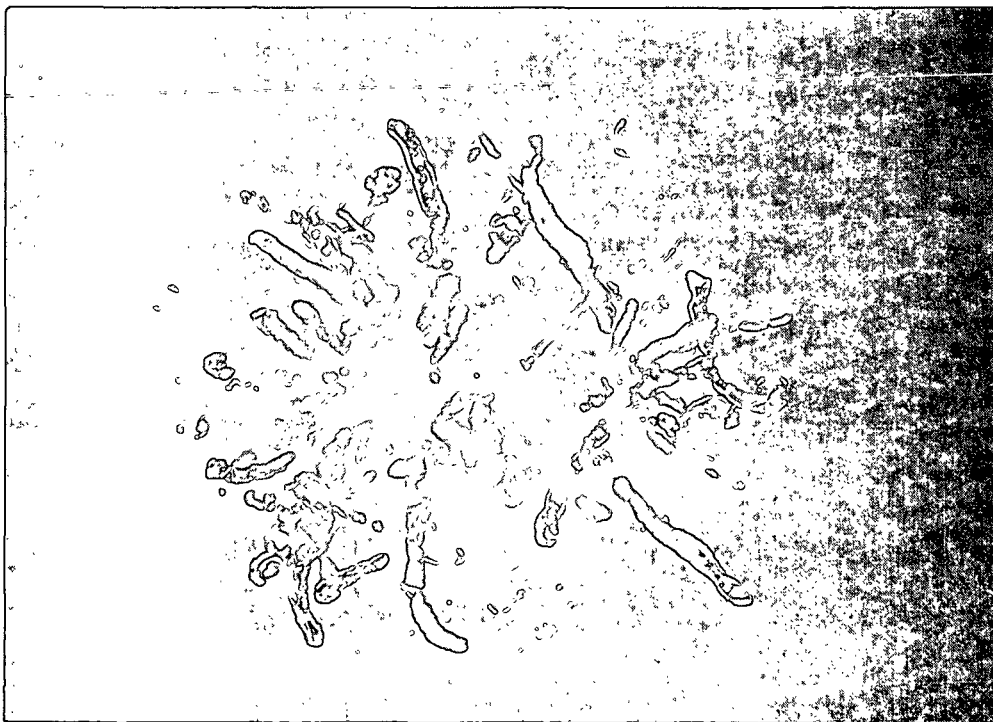


Figure 6. Light Micrographs of Organogenesis in Large Clumps of Douglas-fir Cells in Suspension Cultures. Same Clump in Polarized Light, Showing Secondary-wall Birefringence in Elongated Cells



Figure 7. Light Micrographs of Organogenesis in Large Clumps of Douglas-fir Cells in Suspension Cultures. One Elongated Cell of Same Clump Showing Details of Spiral Thickenings of Secondary Wall

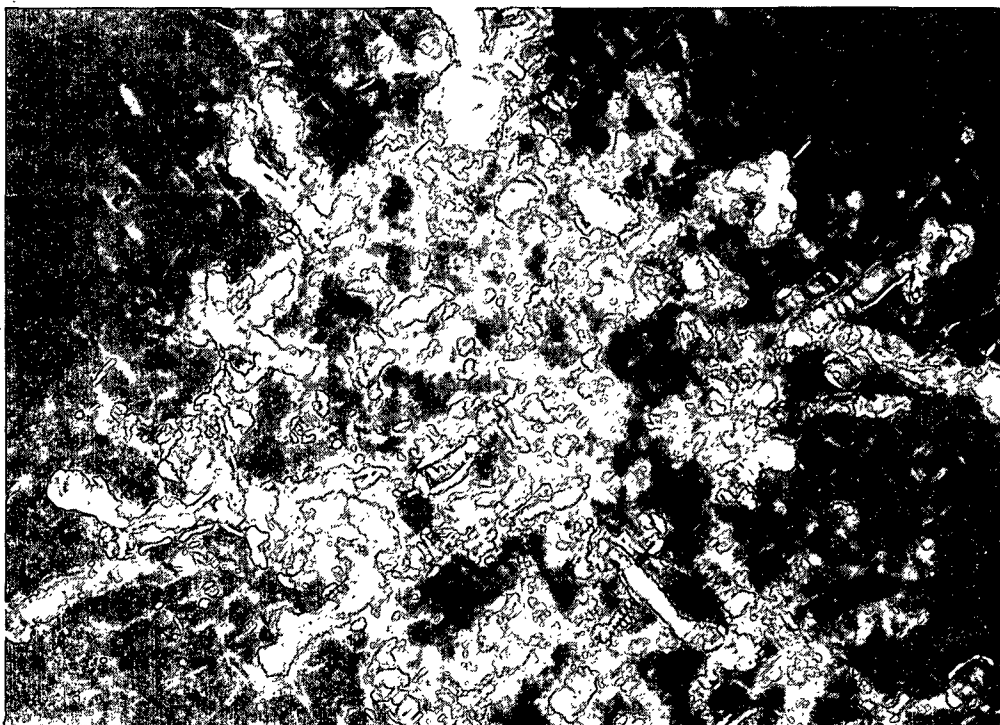


Figure 8. Light Micrographs of Organogenesis in Large Clumps of Douglas-fir Cells in Suspension Cultures. Another Clump Showing Shorter Birefringent Cells, but Some Indication of Parallel Alignment

Other Advances

Prototypes are being designed and built to add a measure of internal monitoring and control to future cultures used in studies of morphogenesis. Some of the subobjectives of the research plan cannot even be approached without first having the means to continuously know and control internal chemical and environmental conditions in our culture vessels. To be sure, many types of automated fermenters are available commercially, but these are expensive and limited funds have forced us into several contingency avenues. This is one area where an investment in a major piece of equipment by an outside agency could have multiple effects in the future toward supplying an industrial prototype for producing the millions of embryos and trees necessary for reforestation.

The success of the main objective is dependent upon the identification, recovery and scale-up of competent cells. But more important, these competent cells must be available from trees 3-5 years old, after their elite traits have been demonstrated. Seeds are not expected from trees so young, and even if they were they would not provide clonal tissue that would be genetically identical with the elite parent. After elite trees are cloned and their ramets crossed with other elite trees under controlled conditions, then clones of seeds may have benefits in propagation, such as preventing monoculture. However, the first step in a large tree-improvement program based on cloning of elite trees demands that a method be used that truly clones elite trees and not their seeds. This is why we have chosen the longer route of mass propagation from suspension cultures, rather than from callus, and in using needle or bud material rather than seed or seedling material as our final callus source. We have deliberately avoided spectacular and quick results with hundreds of shoots from seed callus in favor of mass production of elite trees on the order of hundreds of thousands or even millions per season. Thus, one very important facet of

our current work is screening for competent cells in needles and vegetative buds found on trees.

Plans

We have essentially completed Stages b, f, and l, involving callus production and the initiation and maintenance of suspension cultures. We plan to complete the current definitive transdisciplinary experiment by July and document the degree of organogenesis achieved in nipple flask suspension cultures. Our emphasis will then shift toward the control of embryogenesis in suspensions. This coming year will probably be spent in preliminary experiments in each laboratory, until we obtain repeatable results, culminating in another transdisciplinary experiment to document embryogenesis.

BIOCHEMISTRY

Mission

The biochemistry laboratory has primary missions which fall within two subobjectives of the research plan:

- (5) Development of indicators and change agents for morphogenesis.
- (6) Control over morphogenesis.

Input is provided also for the theoretical basis for morphogenesis, subobjective (4), but that topic will not be addressed in this report.

Factors

The major factors under investigation during this period relative to subobjective (5) were isozymes and endogenous auxin. These are also included under subobjective (6) along with others such as peroxide and phenolics. The latter have received limited attention over the past year. Several additional biochemical parameters were examined for their value as developmental indicators.

Research Highlights

Biochemical indicators would make it possible to assess the potential for morphogenesis of cultured tissue and also to monitor the progress of morphogenesis once initiated. Many biochemical parameters which could be considered in these roles are often of a secondary nature, i.e., indirectly correlated with developmental events but not causative. Secondary parameters may be very good indicators for the progression of morphogenesis but poor subjects for exploitation in the development of change agents which could lead to external control over morphogenesis. As indicated in previous reports we have considered indoleacetic acid oxidase (IAA oxidase) as a causal control point (cf. Progress Report Five, pp. 12-16). Coincident with the current scale-up of suspension cultures, we have examined the critical parameters in the new culture systems. All data presented in this section, except the EDAX data, were derived from Douglas-fir suspension cells and spent media at Step m of Fig. 4. Results of analyses of endogenous auxin are presented under the section, Research Fellows, since Dr. Hsu has been conducting that investigation.

It was earlier recognized (cf. Progress Report One, p. 32; Progress Report Two, pp. 35-38) that conifer cells growing in suspension release biopolymers to the medium. These substances may be important for the survival, growth and development of cells. Their production leads to a clouding of the culture medium. The material causing the cloudiness has been recovered and examined. This "cloudy factor" (CF) consists mainly of precipitated protein although carbohydrate is also present, perhaps along with other substances. Energy dispersive x-ray analysis (EDAX) of CF from loblolly pine shows the presence of elements other than C, H, O, and N, namely, Si, P, S, Ca, and Fe. Removal of both cells and CF leaves clear spent medium (CF supernatant) still containing considerable amounts of extracellular products. Anionic disk electrophoresis

of CF supernatant yields a complex protein pattern (Fig. 9). Thus, CF appears to arise by precipitation from a heterogeneous mixture of proteins. Further protein precipitation can be induced (Fig. 10) from the CF supernatant but not by boiling or trichloroacetic acid (a common protein precipitant).

When Douglas-fir cells are removed from the medium and washed with water simply by resuspension and centrifugation, the resulting wash water shows some UV absorption. However, if these washed cells are stirred gently in water (conditions which seem to result in little cell breakage) UV-absorbing material is continuously leached as shown in Fig. 11. Peroxidase and IAA oxidase can be detected in these leachates and in the CF supernatant (Fig. 12 and 13). We found that in the leachate only the higher molecular weight and less anionic peroxidase was released, possibly suggestive of its association with the cell wall.

Other results which we have obtained from suspension cultures grown in the new nipple flask system are shown in Table I. While the data in the table are essentially of a base line nature, we are conducting these analyses (along with the enzyme analyses, the use of tracers, and other procedures yet to be developed) to monitor the progress of cell cultures. It is anticipated that some of these systems will be automated and incorporated into chemostat systems in the future.

Other Advances

The reorganization of this project meant that some areas of investigation that were ongoing at the time of the last report were temporarily shelved in favor of opportunities presented by the new culture systems. Based upon thin-layer chromatography (TLC), it appears that needle callus phenolics consist mostly of glycosides in contrast to what is encountered in needle extracts. TLC of

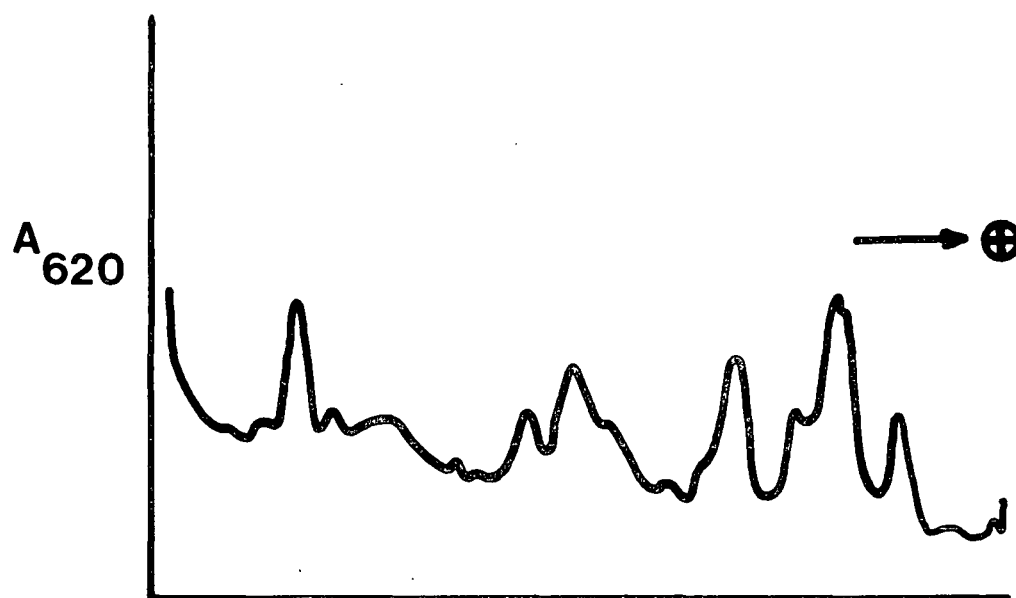


Figure 9. Proteins of Cloudy Factor (CF) Supernatant from Douglas-fir Cell Cultures Resolved by Anionic Electrophoresis

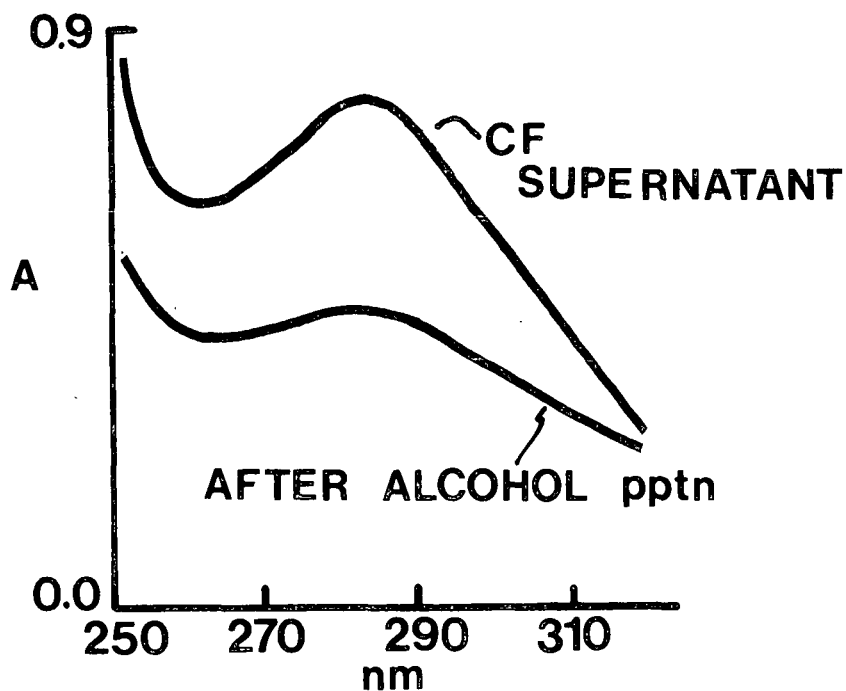


Figure 10. Absorption Spectrum of Cloudy Factor (CF) Supernatant Before and After Alcohol Precipitation

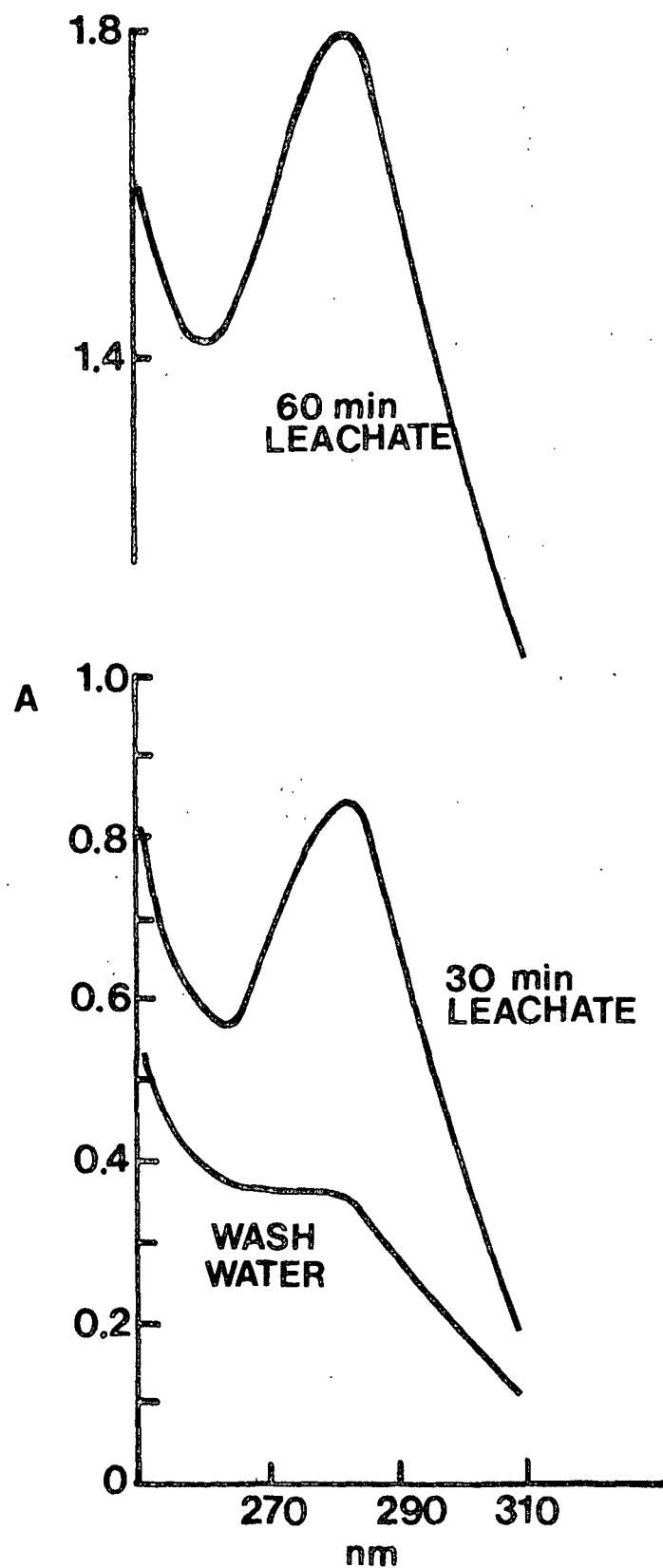


Figure 11. Absorption Spectra of Wash Water and Leachates from Cells

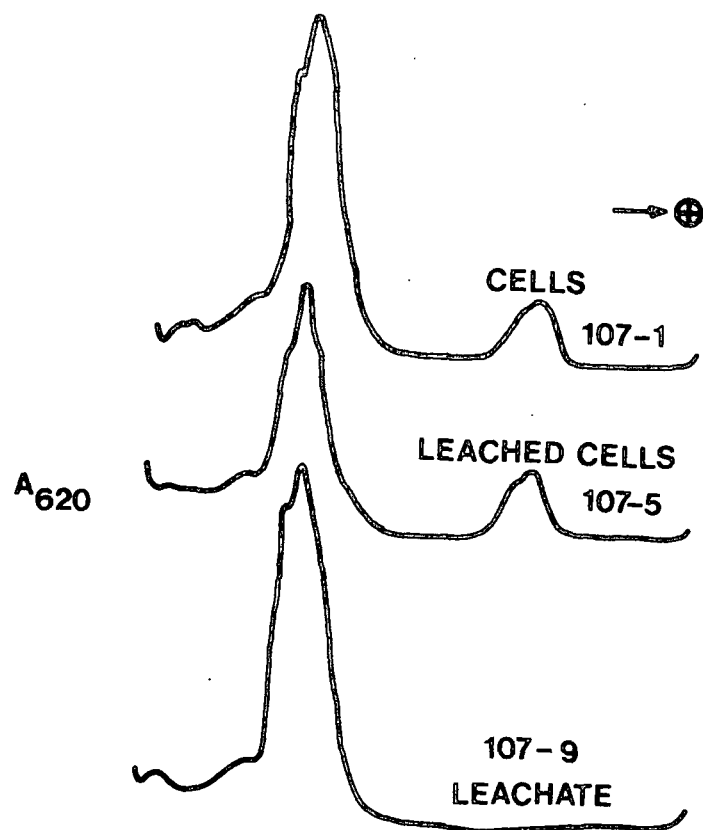


Figure 12. Anionic Electrophoretic Isozyme Patterns of Peroxidase from Cells, Leached Cells and Leachates

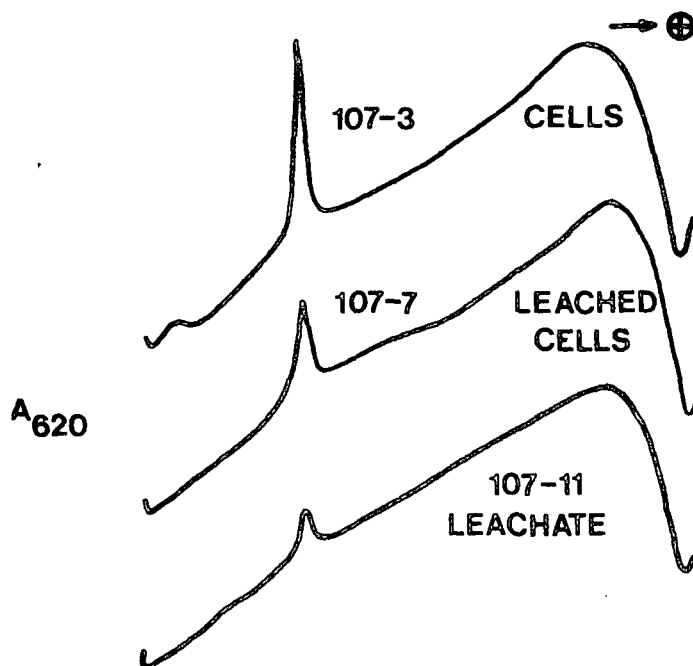


Figure 13. Anionic Electrophoretic Isozyme Patterns of IAA Oxidase from Cells, Leached Cells and Leachates

TABLE I

TERMINAL ANALYSES OF NIPPLE FLASK CULTURES
OF DOUGLAS-FIR AFTER 40 DAYS IN DM MEDIUM

Analytical Protocol ^a	Fraction Analyzed			
	Cells	CF Supernatant	Unused Medium	Other
Fresh wt., mg/cc packed cells	210.4	--	--	--
Dry wt., mg/cc packed cells	6.6	--	--	--
Packed cell volume, cc	29	--	--	--
Freeze-dried CF, mg	--	--	--	13.0
pH	--	4.97	5.6	--
Peroxide, nmoles/cc packed cells	--	3.1	1.5	--
Total carbohydrate, mg glucose equiv./cc packed cells or CF supernatant	15.3	19	35	--
Total phenolics, mg gallic acid equiv./cc packed cells or CF supernatant	10.3	0.0275	--	--
Endogenous O ₂ uptake, μl/min/cc packed cells (in dark)	2.3	--	--	--

^aAll data are averages of triplicates except O₂ uptake (duplicate).

hydrolyzed extracts also suggests the presence of bound indoles. Some known phenolics were found to affect IAA oxidase activity of isozymes which had been resolved on gels by electrophoresis. The new spectrophotometer has allowed us to follow, in the ultraviolet, the course of the IAA oxidase reaction and its perturbations. Unfractionated extracts from different callus sources do not always exhibit the same behavior in these analyses. Gel filtration has shown promise for the fractionation of these extracts if needed.

Plans

Our schedule for the coming year for subobjective (5) calls for further development of indicators and change agents. This means evaluating and retaining or discarding those currently in use as well as investigating new parameters. It should be emphasized that we will not be starting the evaluation of many new parameters. A substantial backlog of information on several agents exists from our earlier perturbation research and current factorial screening of callus. Particular attention will be devoted to natural agents such as the cloudy factor and associated substances released by cells into the medium.

The peroxide regulation and IAA oxidase hypotheses continue to provide high expectations as a means of reaching subobjective (6). Therefore, these enzymes will be examined further so as to modify their behavior in vivo. Also, elite clonal material is being obtained from client companies which should permit better assessment of genetic influences on isozyme patterns. Should measurements of peroxide per se in vitro prove not meaningful, the more difficult in vivo or indirect determinations will be introduced into our plans. The anticipated departure of Dr. Hsu this summer will not signal the end of our work on levels of endogenous auxin. Investigations on the influence of endogenous low molecular weight compounds (principally phenolics as mentioned above under Other Advances) will continue and will be evaluated at the ultrastructural level.

Other attractive avenues of research are open to us that will be probed by tracer studies, e.g., the metabolic flow of carbon and nitrogen sources.

There is no doubt that biochemical control mechanisms that relate to the genetic superiority of the cells in culture will be more fully integrated with the work of our tissue culture specialists and fiber science group. Efforts are underway in this respect.

MORPHOLOGY AND FIBER SCIENCE

Mission/Factors

The morphology and fiber science laboratory has a primary mission:

- (4) Development of a theoretical and structural basis for developmental and morphogenetic events.

Currently, the electron microscope lab functions primarily in a service capacity for light and electron microscopy of culture controls and treatments. Factors of concern to us in this endeavor include cell divisions and wall development, cell organization and aggregation trends, comparison of these trends and features of in vitro embryoids to the development of true seed proembryos and embryos, and the general assessment of cultured cell secretory and storage activities.

Much of the above information is needed at this time to provide morphological markers and standards and to insure continued progress in understanding the developmental biology of cells. Specific subobjectives of the research plan include the development of indicators and change agents and how they relate to the control of morphogenesis of cloned cells in culture. Later, we will be concerned with other subobjectives related to the production and manipulation of protoplasts.

Research

Primarily, we have been concerned with assisting in the documentation and interpretation events leading to differentiation of cells in suspension cultures and to the development of "shoot-like" structures on various callus sources. Perhaps the most interesting finding was the bleb structure on cells of callus from a Douglas-fir hypocotyl source. Earlier in this project, we proposed that perhaps these blebs were indicative of a potential for organogenesis not present in callus without such blebs. Eventually, we hope to either prove or disprove this hypothesis, but currently this phenomenon continues to present an interesting enigma.

Plans

Future work involving the electron microscope lab will be centered on the comparison of in vivo versus in vitro embryogeny of Douglas-fir. Developing ovules with proembryo structures will be harvested this summer and examined via light and electron microscopy to provide a standard by which to judge the in vitro production of Douglas-fir "embryoids." Later on this year, we hope to carry out autoradiography experiments at both the light and electron microscope level. Continuing to monitor control and treatments in the Tissue Culture Lab will also occupy a good deal of our time over the next several months.

RESEARCH FELLOWS

Dr. Francis Hsu

Mission

Dr. Hsu's objective was to analyze cultured and organized tissues of Douglas-fir and loblolly pine for endogenous auxin and relate the results to the IAA oxidase hypothesis. The rationale for this mission was presented on page 103 of Progress Report Five.

Factors

Results from this effort were expected to contribute to subobjectives (4), (5), and (6) of the research plan. The major factor to be determined was the concentration of free IAA as a function of organizational state and also as a function of culture conditions.

Research Highlights

The initial approach to this problem was to use conventional gas liquid chromatographic (GLC) analysis. The literature contained reports of success with this procedure for other plant species. Bioassays were considered as an alternative but rejected, particularly after some preliminary trials indicated that interpretation would be difficult. Eventually, the conventional GLC system was also discarded. The reason was not the GLC itself so much as the substantial and variable losses that occurred during workup of samples prior to injection into the chromatograph.

The isotope derivative assay which is used currently to quantitate endogenous IAA, employs GLC also but in a more sensitive manner. There is only one step prior to derivatization of the sample; losses are therefore minimized and less variable during workup. Once the derivative (the ^{14}C -methyl ester of IAA) is made, carrier can be added as needed to facilitate isolation and purification. It is, however, necessary to isolate the derivative in a pure state before counting. For this purpose two sequential GLC columns are used followed by TLC. Two measurements are then necessary, liquid scintillation counting and quantification of the GLC peak with an internal standard.

Results for needles and two clones of needle callus of Douglas-fir are given in Table II. The IAA content of the callus is higher than that of the originating tissue on a fresh weight basis (it would be still more pronounced

on a dry weight basis). Needle callus clones examined varied substantially in IAA content; furthermore, the level of free endogenous IAA increased with culture age which is consistent with the general observation that old callus is often unsuitable for morphogenesis studies. Relative to the peroxide regulation and IAA oxidase hypotheses, the IAA content is directly correlated with catalase activity. What may heighten the significance of that relationship still further is the fact that clone 42 is competent for morphogenesis (shoot production) while clone 31 has never produced shoots.

TABLE II

FREE IAA CONTENT OF NEEDLE CALLUS AND NEEDLES

Tissue Analyzed	IAA, nonograms per gram fresh weight
Needles, 38-day seedlings	2.1
Needles, 116-day seedlings	2.2
Needle callus Clone 42:	
14 days after 10th subculture	4.9
8 days after 12th subculture	9.0
21 days after 12th subculture	11.9
Needle callus Clone 31:	
14 days after 15th subculture	30.5
3 days after 16th subculture	72.7

Plans

Dr. Hsu's research has produced reliable data only rather recently. It is anticipated that he will be able to analyze several more samples before his departure later on this summer.

Dr. Kimani Waithaka

Mission

Dr. Waithaka's main objective was to develop growth conditions, methodology and nutrient medium to support rapid growth of loblolly pine cells in suspension cultures. Loblolly pine is the most important of the southern pines, and represents trees of high importance to many IPC member companies. Unfortunately, this and other pines are difficult to culture, and Waithaka's efforts produced a breakthrough in continuous cultures and a degree of organogenesis in suspension cultures.

Factors

Results of this study will contribute to subobjectives (2), (3), and (6) of the research plan for loblolly pine (see Tissue Culture section for Douglas-fir results). The major factors were medium composition, environmental control, culture vessels and cell clump size.

Research Highlights

Essentially, all technical advances described for Douglas-fir were also observed in suspension cultures of loblolly pine. High clonal variability permitted only a few needle callus cultures to survive past the first few passages (Stage b, Fig. 4). This confounded early studies that tested several basic media. However, when elite-tree seedling hypocotyl segments were used for explants and callus was used for suspensions before subculturing, both maintenance (Stage l) and organogenic (Stage k) suspensions were obtained. Nipple flasks were superior to test tubes. Using the new source of callus and some fine adjustments in the environment, suspension cultures grew equally well in most media. Thus, MS medium was adopted for general use, giving monthly increases in wet weight of 8-10 fold.

As with Douglas-fir, cells in large clumps differentiated into elements resembling those found in primary elongated vascular strands. Some parallel cell lineages were observed. However, no meristematic areas were observed in either species.

The most encouraging result was the recovery of a rather large proembryo-like structure from a suspension culture, which would be Stage j, embryogenesis. Figures 14 and 15 show the proembryo as seen through the light and electron microscopes.

Plans

Dr. Waithaka terminated his 18 months at the Institute the last of June, 1978. He accepted a teaching and research position at the University of Nairobi, Kenya, where he hopes to establish their first tissue culture laboratory. During June, a new technician learned the methodology for loblolly pine callus and suspension cultures, and will serve as a bridge until we fill the postdoctoral position this fall. The mission of the new post-doctoral fellow will be to continue the work of Dr. Waithaka, and also to develop similar methods for loblolly pine callus from needles and buds of elite trees.

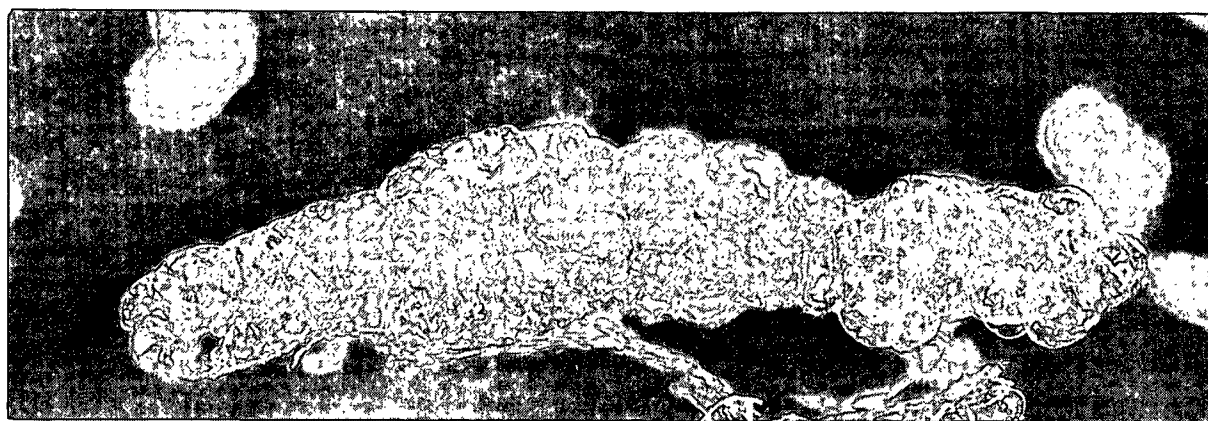


Figure 14. Proembryoid Recovered from a Suspension Culture
of Loblolly Pine



Figure 15. Electron Micrograph of Proembryoid Shown in Fig. 14

PROJECT OUTPUT: SUMMARY

INFORMATION

Annual Report, Project 3223, August 1, 1977

RAC Report, October 28, 1977, March 30, 1978

Publications

1. Durzan, D. J., 1978. Tissue culture - genetic and physiological aspects. p. 137-164. In Proc. 4th North American Forest Biology Workshop, State Univ., New York, College Environmental Sciences and Forestry, Syracuse.
2. Graham, L. L. and Johnson, M. A., 1978. Sucrose synthetase from triploid quaking aspen callus. In press, Phytochemistry Vol. 17. Includes data from cultured conifers.
3. Johnson, M. A. and Carlson, J. A., 1977. Embryoid induction relative to isozymes of peroxidase and IAA oxidase from organs and cultured tissue of Douglas-fir. Plant Physiol. Suppl. 59: 62.
4. Parham, R. A. and Kaustinen, H. M., 1976. Differential staining of tannin in sections of epoxy-embedded plant cells. Stain Technol. 51(4): 237-40.
5. Parham, R. A. and Kaustinen, H. M., 1977. On the site of tannin synthesis in plant cells. Bot. Gaz. 138(4): 465-7.
6. Winton, L. L. and Verhagen, S. A., 1977. Embryoids in suspension cultures of Douglas-fir and loblolly pine. p. 21-24. TAPPI Forest Biology Conf., Madison, June 20-22.
7. Winton, L. L. and Verhagen, S. A., 1977. Shoots from Douglas-fir cultures. Can. J. Bot. 55: 1246-50.
8. Winton, L. L., 1977. Applied and fundamental aspects of plant cell, tissue, and organ culture. Book review, Forest Sci. 23: 419.

The following contain reports contributed by the project.

9. Grummer, A. E., 1977. The development of test tube trees. Paper 187, No. 5.
10. Hanson, J. P., 1977. Studies indicate test-tube trees could be future pulpwood source. Pulp Paper, June.
11. Bylinski, G., 1978. The cloning era is almost here. Fortune, June 19.

Courses

A-377 Forest Genetics

A-378 Plant Tissue Culture

Miscellaneous

Dr. D. J. Durzan visited Weyerhaeuser (Centralia) and Crown Zellerbach (Wilsonville) between April 24 to 28 to discuss client-oriented requirements for the tissue-culture-genetics project.

TECHNOLOGY

Representatives of member companies are welcome to see the newly-built culture apparatus at The Institute of Paper Chemistry. A preview to the executives of member companies was given during the Executives' Conference May 9-10 in Appleton.

MANPOWER

Dr. K. Waithaka has accepted a position of Lecturer at the University of Nairobi, Department of Crop Science, Nairobi, Kenya. He will start teaching and research in October, 1978.

Dr. F. Hsu will complete his postdoctoral assignment, started May, 1977, this summer.

A prospective postdoctoral candidate to replace Dr. Waithaka has been identified and we expect a letter of acceptance this summer.

THE INSTITUTE OF PAPER CHEMISTRY

A handwritten signature in cursive script, appearing to read 'D. J. Durzan', is written over a horizontal line.

D. J. Durzan
Group Leader
Biochemical Systems
Forest Biology Section

REPLY SHEET FROM MEMBER COMPANIES

Please address replies to: Dr. D. J. Durzan
The Institute of Paper Chemistry
P. O. Box 1039
Appleton, Wisconsin 54912

Comments:

Request for more information:

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|-----------------------------------|--------------------------|
| 1. Project 3223 | <input type="checkbox"/> |
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| Technology | <input type="checkbox"/> |
| Manpower | <input type="checkbox"/> |
| 3. How can my company contribute? | <input type="checkbox"/> |

